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# Activation of Coagulation and Angiogenesis in Cancer

## *Immunohistochemical Localization* in Situ *of Clotting Proteins and Vascular Endothelial Growth Factor in Human Cancer*

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**Thrombin-catalyzed, cross-linked fibrin (XLF) formation is a characteristic histopathological finding in many human and experimental tumors and is thought to be of importance in the local host defense response. Although the pathogenesis of tumor-associated fibrin deposition is not entirely clear, several tumor procoagulants have been described as likely primary stimuli for the generation of thrombin (and XLF) in the tumor microenvironment (TME). In a previous study of a variety of human tumors we have shown that tissue factor (TF) is the major procoagulant. However, the relative contribution to fibrin deposition in the TME of tumor cell TF and host cell TF (eg, macrophage-derived) was not established. In addition, recent evidence has implicated TF in the regulation of the synthesis of the pro-angiogenic factor vascular endothelial growth factor (VEGF) by tumor cells. In the current study we used *in situ* techniques to determine the cellular localization of XLF, TF, VEGF, and an alternative tumor procoagulant, so-called cancer procoagulant (CP), a cysteine protease that activates clotting factor X. In lung cancer we have found XLF localized predominantly to the surface of tumor-associated macrophages, as well as to some endothelial cells and perivascular fibroblasts in the stromal area of the tumors co-distributed with TF at the interface of the tumor and host cells. Cancer procoagulant was localized to tumor cells in several cases but not in conjunction with the deposition of XLF. TF and VEGF were co-localized in both lung cancer and breast cancer cells by *in situ* hybridization and immunohistochemical staining. Furthermore, a strong relationship was found between the synthesis of TF and VEGF levels in human breast cancer cell lines ($r^2$ = 0.84; $P < 0.0001$). Taken together, these data are consistent with a highly complex interaction between tumor cells, macrophages, and endothelial cells in the TME leading to fibrin formation and tumor angiogenesis. *(Am J Pathol 1998, 152:399–411)***

Shortly after the clinical observations of Trousseau in 1865,[1] who first postulated a link between migratory thrombophlebitis and the presence of an otherwise silent malignancy, Billroth[2] described the frequent pathological finding of postmortem blood clots and fibrin-like material in many tumor types. This latter observation has been confirmed in more recent studies using highly specific probes for fibrin[3] and has stimulated a renewed interest

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in the pathogenesis of fibrin deposition in human malignancy and the clinical clotting abnormalities observed in some of these patients.[4,5] Although the precise sequence of events remains uncertain, suggested mechanisms have included direct activation of coagulation by tumor cells in the bloodstream (contributing to the characteristic hypercoagulable state of cancer), activation at extravascular sites of solid tumor growth, and/or indirect activation of clotting by host inflammatory cells responding to tumor products.[3–6]

Extravascular deposition of fibrin within the tumor microenvironment (TME) of solid tumors was first detailed by O'Meara.[7] However, the significance of this association was not generally appreciated, partly because of difficulties in the histological identification of fibrin in tissue sections and partly because immunohistological approaches failed to discriminate between fibrin, fibrinogen, fibrin degradation products (FDPs), or fibronectin, all of which can be found in tumor stroma.[8,9] These difficulties have been overcome to some extent through the biochemical evaluation of extracts of solid tumors from experimental animals and by the use of monoclonal antibodies (MAbs) that react specifically with cross-linked fibrin (XLF) but not with fibrinogen, FDP(s), or fibronectin.[3,6–9]

Extravascular fibrin deposition around solid tumors would appear to require at least two conditions. First, disruption of the usual vascular permeability barriers must occur, allowing fibrinogen and other required coagulation protein substrates access to the extravascular space. In at least some human and experimental tumors this process may be mediated by tumor production of the cytokine vascular endothelial growth factor (VEGF; formerly known also as vascular permeability factor)[8–11] and/or macrophage production of tumor necrosis factor (TNF).[12] Second, fibrinogen must be cleaved by thrombin. Some evidence exists that VEGF and TNF may be capable of supporting both functions, as both cytokines are capable of activating the procoagulant tissue factor (TF) in endothelial cells and macrophages,[11,12] resulting in the local production of thrombin. Conversely, fibrin formed by this process can induce TF expression by endothelial cells,[13] and both thrombin and fibrin have been shown to be pro-angiogenic.[14,15] More recently, it has been demonstrated in murine tumor models that TF up-regulates VEGF expression by a clotting-independent mechanism, hence enhancing micro-blood vessel formation and diffusion of blood-clotting proteins into the TME.[16]

Analysis of extracts from a variety of human and experimental tumors have implicated several procoagulant molecules in the initiation of tumor-associated coagulation.[17,18] Included prominently among these procoagulant molecules are TF (also known as thromboplastin or factor III), a 47-kd cell surface transmembrane glycoprotein and high-affinity receptor for factors VII and VIIa, and cancer procoagulant (CP), a 68-kd factor-VII-independent cysteine proteinase, which directly activates factor X. The generation of thrombin, stimulated by either of these tumor procoagulants, is greatly enhanced by cell surface assembly of the prothrombinase complex,[19] an event that can be shown to occur on the surface of platelets, tumor cells, and tumor-associated host macrophages.[19,20] Thus, it is possible that a variety of properties of tumor cells and reactive host cells conspire to activate clotting, generate fibrin, and stimulate angiogenesis in the TME. Our *in situ* observations in this study provide additional support for the importance of TF in tumor cells and macrophages in the pathogenesis of fibrin formation and promotion of tumor angiogenesis in several tumors, but most convincingly in human lung cancer.

## Materials and Methods

### Cells and Tissues

Biopsy specimens from 13 patients in a companion study[21] were too small to provide meaningful functional data and, therefore, were processed only for immunohistology. Of these samples, nine proved technically adequate for interpretation, including malignant effusions from two patients with breast cancer, four patients with ovarian cancer, two patients with melanoma, and one patient with lung cancer. The results of the immunohistological studies on patients in this previous series, in which correlative functional data was available,[21] provided the impetus for a more detailed, prospective immunohistological study. Therefore, the current study was undertaken of fresh surgical specimens of 25 consecutively accessioned malignant lung tumors, 4 normal lung tissues, and cells from a malignant pleural effusion from a patient with squamous cell carcinoma of the lung. These tissues were obtained at the time of lung resection, open biopsies, or in the case of normal tissues, at postmortem. The lung tumors for this study included squamous cell carcinoma (n = 11), adenocarcinoma (n = 7), large-cell carcinoma (n = 5), and small-cell carcinoma (n = 2).

For histological studies, tissues were subdivided and fixed in formalin for paraffin embedding and routine histology or quick frozen in isopentane and liquid nitrogen (as noted below) and stored at −80°C for subsequent immunohistology. Effusions were collected aseptically and stored in glass bottles at room temperature for 1 to 4 hours before processing. The solid tumors were placed in Hanks' balanced salt solution (HBSS) at the time of surgery and stored at 4°C for 1 to 6 hours before processing. Additional specimens of invasive ductal breast cancer and lung cancer for analysis by immunohistochemistry and *in situ* hybridization were obtained from Dr. Toncred Styblo, Winship Cancer Center, and Dr. Cynthia Cohen, Department of Pathology, Emory University, and were processed as described in detail elsewhere[22] and *vide infra*. Cytospin smears of pleural or peritoneal cells were examined after Wright stain or immunoperoxidase labeling.

### Antibodies for Immunohistochemistry

The MAbs against coagulation proteins used in this study and their specificities are listed in Table 1.[23–31] Briefly,

**Table 1.** Characterization of Monoclonal Antibodies Directed against Coagulation Proteins

| Antibody | Specificity | Reference or commercial source |
|---|---|---|
| 1-8C6 | Fibrinogen and fibrin I (Bβ1–42) | Kudryk et al,[23] |
| T2G1 | Fibrin II | Kudryk et al,[24] |
| GC4, 2G10-1 | Fibrin degradation products | Kudryk et al,[25] |
| UC-45 | Cross-linked fibrin | Hogg[26] |
| 2C4, 5G9 | Tissue factor | Morrissey et al,[27]<br>Ruf et al,[28] |
| A1-3 | Tissue factor | Ewan et al,[29]<br>Hair et al,[30] |
| 4509 | Tissue factor | American Diagnostica, Greenwich, CT |
| Anti-CP | Cancer procoagulant | Gordon et al,[31] |

All MAbs are of the IgG1 subclass, except for A1-3, anti-CP, and UC-45, which are IgM MAbs, and 1-8C6, which is an IgG2a MAb.

the 1-8C6 MAb, which is specific for fibrinogen or fibrin I (des-fibrinopeptide A-type fibrin) but not fibrin II (des-fibrinopeptide B-type fibrin),[23] requires an intact 14 Arg-15 Gly bond on the Bβ chain of fibrinogen for reactivity. Anti-fibrin MAb T2-G1 reacts with the amino-terminal part of the Bβ chain only after removal of fibrinopeptide B (FPB, Bβ 1 to 14) by thrombin, and hence binds to fibrin but not fibrinogen.[24] The CG4 MAb reacts with fragment D of fibrinogen and D-dimer derived from cross-linked fibrin but not with either fibrinogen or non-cross-linked fibrin.[25] The MAb UC-45, which is directed specifically against the α-chain of XLF,[26] was a generous gift of Dr. Nancy Hogg (Imperial Cancer Research Fund, London, UK).

The anti-TF MAbs 2C4 and 5G9 inhibit assembly of factors VII and VIIa with TF, and 5G9 also inhibits the function of the TF-VIIa complex.[27,28] The MAb 4509, which was obtained from American Diagnostica (Greenwich, CT), also blocks VII binding to TF. The MAb A1–3[29] recognizes and inhibits the procoagulant activity (PCA) of TF expressed on the surface of activated monocytes and macrophages and recognizes both purified human brain TF and recombinant TF on Western blots.[30] It should be noted that the normal tissue distribution of antigens reactive with MAb A1–3, as compared with 2C4 and 5G9, are somewhat different.[32,33] This implies that A1–3 may cross-react with epitopes of proteins other than TF. However, unless indicated otherwise in the description of the immunohistochemical results, the distribution of TF antigens recognized by all three MAbs was identical, as determined by two blinded observers. The anti-CP MAb is an IgM that was developed by standard methodology and used in an ELISA for the analysis of CP in the serum of cancer patients.[31] This MAb does not block CP enzymatic activity. Unlike the antibodies against TF, no extensive immunohistological profile is available for the distribution of CP using the CP MAb.

Additional antibodies utilized included an anti-cytokeratin MAb and MAb markers for the following leukocyte populations obtained from Dako (Santa Barbara, CA): all leukocytes (anti-leukocyte-common antigen), T cells and T cell subsets (T1, T4, and T8), and monocytes/macrophages (EBM-11 and MAC-120). Cells bearing interleukin-2 receptors were detected using the anti-Tac MAb, a generous gift of Dr. Thomas Waldman (National Institutes of Health, NIH, Bethesda, MD). Mouse MAb anti-human CD31, which recognizes human CD31 on endothelial cells, was obtained from Dako. Isotype control MAbs were obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit antisera to von Willebrand factor (vWF) and fibrinogen/fibrin were obtained from Dako. Anti-VEGF (Ab-2) (Oncogene Science, Cambridge, MA) is a rabbit polyclonal antibody (PAb) directed at the amino terminus region of human VEGF. Biotinylated horse anti-mouse immunoglobulin (Ig), biotinylated goat anti-rabbit Ig, and avidin-biotin-peroxidase complexes (ABC) were purchased from Vector Laboratories (Burlingame, CA).

## *Labeling*

Procoagulant molecules and cell antigens were localized in cryostat sections or cytospin smears using MAbs or PAbs and an ABC-peroxidase method.[34] Optimal working dilutions of antibodies were determined by preliminary evaluation of human renal allograft rejection,[35] term placentae, and lipopolysaccharide-treated monocytes.[32] Briefly, paraformaldehyde-fixed cryostat sections were incubated overnight at 4°C with MAb or PAb, followed by biotinylated horse anti-mouse Ig or biotinylated goat anti-rabbit Ig (5 μg/ml) and ABC complexes. Sections were then incubated with the substrate diaminobenzidine, counterstained with hematoxylin, dehydrated, and mounted. Each tissue was labeled with an isotype-matched negative control MAb (25 μg/ml), and endogenous peroxidase was blocked by the addition of sodium azide to the substrate solution.[34]

## *Analysis of Immunohistological Labeling*

The results of immunohistological labeling were assessed independently in a blinded fashion by two pathologists, focusing on the binding of antibodies to tumor cells *versus* adjacent stromal cells (leukocytes, endothelial cells, and fibroblasts) or connective tissue. For each tumor, results were classified into those with extensive tumor or stromal labeling (+), weak or very focal (+/−), or no labeling (−), using the MAbs listed in Table 1. Positive immunohistological results were assessed by the $\chi^2$ test for a statistically significant association between the presence of a given procoagulant molecule and correspond-

ing XLF deposits. The level of agreement between the two observers was virtually 100%.

## In Situ *Labeling of Tissue Factor with Recombinant Factor VIIa*

In an effort to confirm that the TF labeled with MAbs was functional and capable of activating blood coagulation, we analyzed some of the tumors with a functional *in situ* probe, biotinylated Phe-Pro-Arg-chloromethyl-ketone-labeled recombinant factor VIIa (FPR-ck-rVIIa). This novel probe, the synthesis, characterization, and application of which has been described in detail elsewhere,[22,36] binds with high affinity to cellular TF as a specific ligand. The recombinant factor VIIa, used to construct the probe, was a generous gift of Dr. Ulla Hedner, Novo Nordisk, Copenhagen, Denmark.

## In Situ *Hybridization*

### *Method 1*

We examined lung cancer tissue for the co-localization of TF and VEGF mRNA using antisense $^{35}$S-labeled riboprobes as described previously.[37–39] Briefly, cryosections were pretreated with paraformaldehyde, proteinase K (Sigma Chemical Co.) and prehybridized in 100 μl of hybridization buffer (50% formamide, 0.3 mol/L NaCl, 20 mmol/L Tris, pH 8.0, 5 mmol/L EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 10% dextran sulfate, and 10 mmol/L dithiothreitol) at 42°C. Serial sections were hybridized with $6 \times 10^5$ cpm of $^{35}$S-labeled riboprobes at 55°C. After hybridization, the sections were washed with 2X SSC (1X SSC contains 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0) with 10 mmol/L β-mercaptoethanol and 1 mmol/L EDTA, treated with RNase A (Sigma), washed in the same buffer, followed by a high-stringency wash in 0.1X SSC with 10 mmol/L β-mercaptoethanol and 1 mmol/L EDTA at 55°C. The slides were then washed in 0.5X SSC and dehydrated in graded alcohols containing 0.3 mol/L $NH_4Ac$. The sections were dried, coated with NTB2 nuclear track emulsion (International Biotechnologies, New Haven, CT), and exposed in the dark at 4°C for 4 to 12 weeks. After development, the sections were counterstained with hematoxylin and eosin (H&E) to aid in cell identification.

The TF cDNA for these studies was a 1.3-kb probe that included the entire coding sequence of human TF as described previously.[38] The VEGF riboprobe was a 518-bp fragment containing the entire VEGF coding sequence. It was synthesized from a reverse transcript of total mRNA from a patient with acute promyelocytic leukemia (enrolled in Eastern Cooperative Oncology Group Study EST 2481 and supplied graciously by Dr. Elisabeth Paietta, Albert Einstein College of Medicine, Bronx, NY). DNA oligonucleotides were synthesized to allow amplification. The 5′ primer sequence was 5′-CACCATGCCAAGTGGTCCCAGGCTGC and the 3′ sequence was 5′-CCGCCTCGGCTTGTCACATCTGCA. For reverse transcription, 1 μg of total RNA was added to 20 μl of PCR Master (Boehringer Mannheim, Indianapolis, IN) diluted according to the manufacturer's instructions. This mixture was heated to 70°C for 7 minutes and then quickly chilled. Fifty nanomoles of the 3′ primer and 10 U of recombinant M-MULV reverse transcriptase (Boehringer Mannheim) were added, and the mixture was incubated in a Perkin Elmer 9000 thermocycler (Perkin Elmer, Norwalk, CT) for 60 minutes at 42°C. The mixture was then heated to 94°C for 2 minutes to inactivate the reverse transcriptase, and 50 nmol of the 5′ primer was added. The fragment was amplified by 35 cycles of polymerase chain reaction (PCR); cycling conditions were 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. This procedure yielded a single band on agarose gel electrophoresis. To allow labeling of the fragment, SP6 and T7 promoter sequences were attached to the 5′ and 3′ ends of the fragment. Oligonucleotides were constructed that contained the promoter sequences at the 5′ end and a 15-bp overlap with the original primers at the 3′ end. To attach these to the original PCR fragment, 50 nmol of each primer was added to 97 ml of diluted PCR Master along with 1 μl of the original amplification mix, and 35 cycles of amplification were performed as described above. As a result, a sense strand could be made using the SP6 promoter, and an antisense strand could be made using the T7 promoter. The fragment was purified by electrophoresis on a 5% denaturing polyacrylamide gel and eluted from the gel by incubation in 300 μl of elution buffer (Ambion, Austin, TX) at room temperature for 16 hours and then used for transcription. The gel was removed and the fragment was precipitated by the addition of 600 μl of 95% ethanol. The gel fragment was recovered by centrifugation for 30 minutes at 4°C and then quickly dried after removal of the supernatant. The gel fragment was reconstituted in nuclease-free water and used as a template for subsequent probe labeling. The cDNAs were transcribed[40] using RNA polymerases in the presence of [$^{35}$S]UTP (Amersham, Arlington Heights, IL; specific activity, 1200 Ci/mmol). Full-length antisense transcripts were used for hybridizations.

*In situ* hybridization experiments using this method were controlled by hybridizing serial sections with the same cDNA probes transcribed in the sense orientation. Each *in situ* hybridization experiment was performed in triplicate on serial sections with the TF and VEGF [$^{35}$S]UTP-labeled sense and antisense riboprobes and developed after a 4-, 8-, or 12-week exposure. This allowed direct comparison of hybridization results obtained with these probes for each tissue.

### *Method 2*

We examined invasive ductal breast carcinoma tissue for the presence of TF mRNA with the *in situ* hybridization and detection system utilized according to the manufacturer's directions (GIBCO-BRL Life Technologies, Grand Island, NY). Slides with paraffin-embedded tissue sections were baked at 65°C for 1 hour, followed by deparaffinization using two changes of xylene. Slides were air dried for 10 minutes after ethanol treatment and then treated with 40 mg/ml protein-

**Table 2.** Breast Cancer Cell Lines

| Cell Line | Characteristics | ER status | Support media |
|---|---|---|---|
| BT-549T | Papilary, invasive ductal carcinoma | NA | RPMI 1640 |
| ZR-75-1 | Infiltrating ductal carcinoma | + | RPMI 1640 |
| BT-474 | Invasive ductal carcinoma | NA | RPMI 1640, containing 10 μg/ml bovine insulin |
| T-47D | Infiltrating ductal carcinoma | + | RPMI 40 containing 10 μg/ml bovine insulin |
| MCF-7 | Adenocarcinoma | + | Eagle's MEM, containing NAA, Earle's BSS, 1 mmol/L sodium pyruvate, and 10 μg/ml bovine insulin |
| MCF-Kei | Adenocarcinoma | + | Eagle's MEM, containing NAA, Earle's BSS, 1 mmol/L sodium pyruvate, and 10 μg/ml bovine insulin |
| BT-20 | Adenocarcinoma | NA | Eagle's MEM, containing NAA and Earle's BSS |
| SK-BR-3 | Adenocarcinoma | NA | McCoy's 5a DMEM, containing 4.5 g/L glucose and 10 μg/ml bovine insulin |
| Hs578T | Ductal carcinoma | − | |
| MDA-MB-231 | Adenocarcinoma | NA | Leibovitz's L-15 |

All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). ER, estrogen receptor (+, positive; −, negative; NA, not available); MEM, minimal essential medium; NAA, non-essential amino acids; BSS, balanced salt solution; DMEM, Dulbecco's modified Eagle's medium.

ase K (GIBCO-BRL) in PBS at 37°C for 5 minutes. Longer proteinase K treatment resulted in decreased signal. Slides were then treated with 4% paraformaldehyde and dehydrated through a graded ethanol series. The slides were hybridized overnight at 42°C and then washed with 0.2X SSC (containing 30 mmol/L NaCl and 3 mmol/L sodium citrate, pH 7.0). The hybridization probe was a mixture of five nonoverlapping biotinylated 20-mer oligonucleotides specific for TF synthesized by R&D Systems Europe, (Abingdon, UK). As a positive control, a reaction using biotinylated oligonucleotides specific for β-actin was performed in parallel. As a negative control, the same tissue was treated with a biotinylated oligonucleotide probe for adenovirus 2. After blocking, the slides were treated with streptavidin-alkaline phosphate conjugate, washed, and treated with substrate nitro blue tetrazolium 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) for 1 hour at 37°C. Slides were then washed in water and dried in ethanol.

### *ELISAs*

Human breast cancer cell lines (Table 2), obtained from the American Type Culture Collection (ATCC, Rockville, MD), were maintained in appropriate media containing 10% fetal bovine serum and 100 U of penicillin and 100 μg of streptomycin per ml. Before assay, $10^6$ cells/10 ml of medium per T-25 flask in triplicate were cultured in a humidified incubator for 4 days at 37°C in 5% $CO_2$/95% air. For VEGF determinations, culture supernatants were harvested and assayed utilizing the ELISA from R&D Systems (Minneapolis, MN), according to the manufacturer's direction. In preliminary experiments, it was demonstrated that all of the cellular VEGF was secreted (results not shown). For TF determination, cells were harvested after incubating with 0.25% trypsin and washed in medium. Finally, cells were resuspended at $10^6$ cells/ml of PBS, sonicated (Ultrasonics Processor, Utrasonics, Inc., Plainville, NY, model GE50, at setting 40 for 20 seconds at 4°C) and assayed for TF antigen by ELISA (American Diagnostica) according to the manufacturer's direction. As virtually none of the TF was lost into the culture supernatant, supernatants were not assayed routinely.

The data were subjected to linear regression analysis using the Sigma Plot program.

## *Results*

Thirteen malignant effusions were processed by cytocentrifugation for immunocytochemical characterization in the initial study. In nine of nine technically adequate specimens, tumor-associated macrophages (TAMs) were plentiful in each specimen, as visualized with the MAb EBM-11 (a representative example is shown in Figure 1). Although tumor cells were easily distinguished morphologically (Figure 1A), or by using a MAb to cytokeratin (data not shown), only the TAMs reacted with the MAbs to TF (Figure 1, C and D). None of the effusions contained tumor cells that reacted with either of the MAbs to TF or the MAb to CP. Stimulated by this preliminary data, we extended our study to solid tumors, concentrating predominantly on lung cancer.

Preliminary evaluation of sections of normal lung tissue showed an absence of reaction with anti-fibrin, -TF, and -CP MAbs (data not shown). All sections of lung tumors contained clusters of tumor cells separated by fibrous stroma. No labeling of tumor sections was seen using isotype-matched control MAbs. A summary of the results on our series of lung tumors probed with MAbs to XLF and the procoagulant molecules TF and CP is presented in Table 3 and described in more detail below.

### *Adenocarcinoma of the Lung*

Four of seven cases of adenocarcinoma of the lung (57%) showed deposits of XLF. In each case, these XLF deposits were confined solely to stromal areas; two cases showed extensive deposit on and adjacent to infiltrating leukocytes, and in two cases XLF deposits were limited to endothelial or perivascular labeling. TF was detected in four of the seven

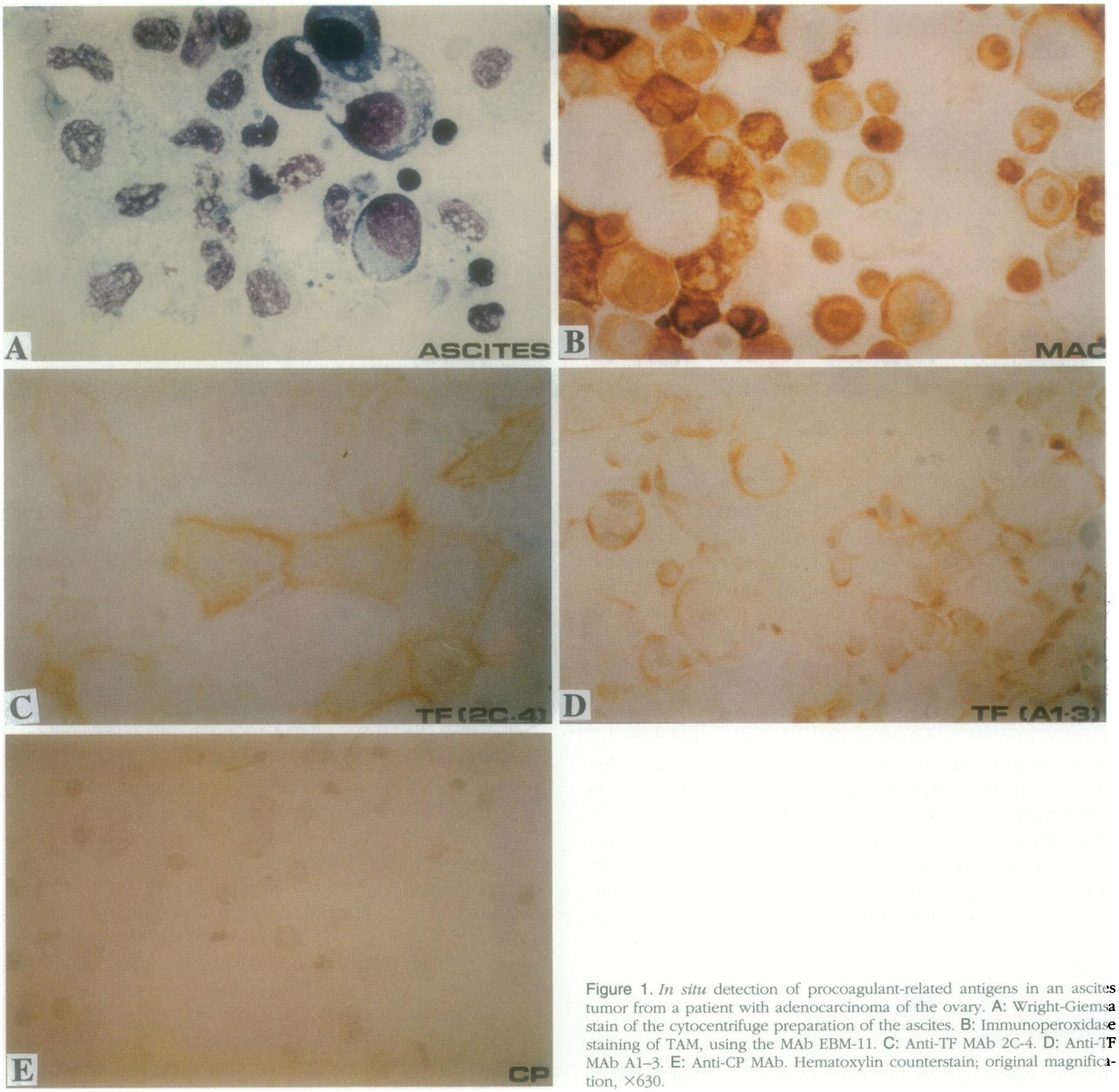


Figure 1. *In situ* detection of procoagulant-related antigens in an ascites tumor from a patient with adenocarcinoma of the ovary. A: Wright-Giemsa stain of the cytocentrifuge preparation of the ascites. B: Immunoperoxidase staining of TAM, using the MAb EBM-11. C: Anti-TF MAb 2C-4. D: Anti-TF MAb A1–3. E: Anti-CP MAb. Hematoxylin counterstain; original magnification, ×630.

adenocarcinoma cases, coinciding with the observed distribution of XLF in three of the four fibrin-positive cases. A representative example is illustrated in Figure 2; an intense, focal reaction of tumor cells with the anti-TF MAb was observed (Figure 2A), with a similar strong and extensive association of TF seen with stromal macrophages (Figure 2, C and D) and vascular endothelial cells (Figure 2F), identified by co-localization with CD31 MAb (Figure 2E). Cancer procoagulant was not localized to any tumor cells; rather, CP was observed in association with perivascular cells, and the anti-CP MAb stained diffusely stromal cells in two cases (data not shown). Extensive and diffuse stromal deposition of fibrinogen and FDPs are observed in almost all cases. As noted in Figure 2, G and H, XLF often defined the TAMs.

In an effort to understand better the potential significance of TF expression in tumor-associated vascular endothelial cells, we also examined a few tumors for co-localization of TF and VEGF, the latter a likely mediator of new blood vessel development. As noted in Figure 2B,

**Table 3.** Summary of Lung Tumor Labeling Using Monoclonal Antibodies to Fibrin and the Procoagulant Molecules Tissue Factor and Cancer Procoagulant

| Histology | Number of cases | Tumor cells | | | Stromal cells | | |
|---|---|---|---|---|---|---|---|
| | | TF | CP | Fibrin | TF | CP | Fibrin |
| Squamous cell | 11 | 4 | 2 | 2 | 9 | 2 | 9 |
| Adenocarcinoma | 7 | 0 | 0 | 0 | 4 | 2 | 4 |
| Large cell | 5 | 3 | 1 | 2 | 4 | 1 | 5 |
| Small cell | 2 | 0 | 0 | 0 | 2 | 0 | 2 |
| Totals | 25 | 7 | 3 | 4 | 19 | 5 | 20 |

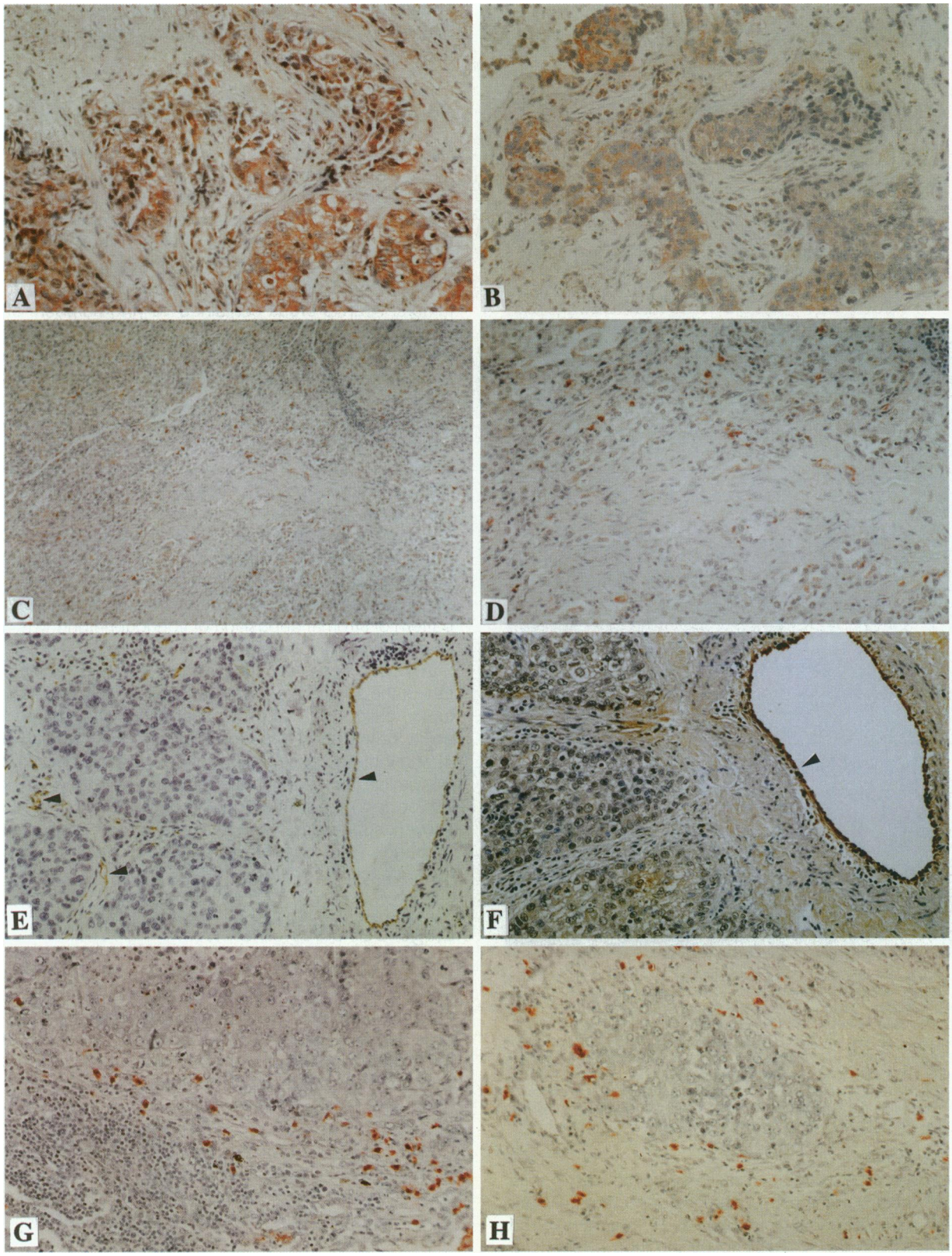


**Figure 2.** *In situ* localization of TF, VEGF, and XLF in human adenocarcinoma of the lung. Fresh tumor was fixed in Bouin's or formalin and probed with mouse anti-human MAbs and the immunoperoxidase technique described in the Materials and Methods. **A:** TF, localized to nests of tumor cells. Magnification, ×50 **B:** VEGF, co-localized to the lung cancer cells with TF. ×50. **C** and **D:** TF, noted principally on the surface of macrophages both within the tumor nodules and more intensely at the periphery in the interstitial inflammatory reaction to the tumor. ×25 and ×50, respectively. **E** and **F:** CD31 and TF localized to tumor blood vessel endothelium. **Arrowheads** indicate positive staining of blood vessel endothelial cells. ×50. **G** and **H:** XLF limited to staining of macrophages in the interstitial, inflammatory infiltrate. ×50.

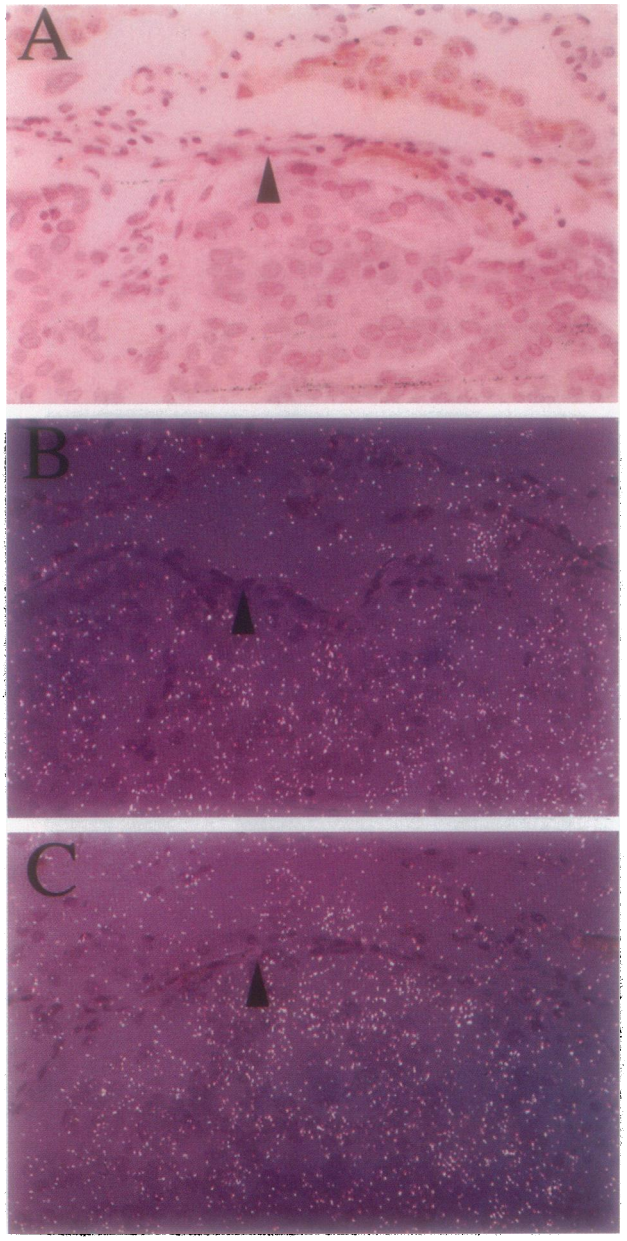


**Figure 3.** Localization of TF and VEGF mRNAs in adenocarcinoma of the lung using *in situ* hybridization. *In situ* hybridization (method 1) was performed with either $^{35}$S-labeled TF (**B**) or VEGF (**C**) riboprobes, as described in Materials and Methods. **Arrowheads** indicate the tumor capsule and the border of the normal lung (above) and tumor tissue (below). TF mRNA was localized primarily to the tumor and not in the normal lung tissue or tumor capsule. VEGF mRNA (**C**) was expressed in the tumor cells and was also found in scattered endothelial cells within the normal lung (not shown). A bright-field photomicrograph of a H&E-stained section of the same region of the tumor and adjacent normal lung is shown in **A** for comparison.

a representative example of six tumors examined to date, VEGF stained prominently in the nests of tumor cells and was found in the same distribution as TF (Figure 2A). That TF and VEGF are synthesized by the lung adenocarcinoma cells is demonstrated by the results of *in situ* hybridization experiments illustrated in Figure 3. Both TF (Figure 3B) and VEGF (Figure 3C) mRNA localized strongly to the tumor nodule of the same adenocarcinoma specimen as illustrated in Figure 2 but were not visualized to any significant degree in either the tumor capsule or the normal adjacent lung tissue (Figure 3, B and C).

## *Squamous Cell Lung Carcinoma*

Deposits of XLF were detected in 9 of 11 cases of squamous cell carcinoma (82%), particularly within stromal areas, whereas only 2 examples of fibrin-positive tumor cell labeling (18%) were observed. Fibrin was commonly observed contiguous to macrophage-like stromal cells and endothelial cells. Anti-TF MAb labeling of cells was also observed predominantly in stromal areas. In most tumors, stromal areas contained varying numbers of infiltrating leukocytes, including 50 to 80% macrophages (EBM-11 positive) and 10 to 20% $CD8^+$ T cells, and endothelial cells in close association with deposits of XLF ($P = 0.001$ by $\chi^2$ analysis). In contrast to the extensive stromal cell reactivity for XLF and TF, few cases showed more than a trace, or very minor, tumor cell reactivity for XLF (n = 2, 18%) or TF (n = 4, 36%). Only two tumors displayed actual tumor cell reactivity with anti-CP MAb. In each case, tumor cells expressed both CP and TF in conjunction with deposits of XLF. Fibrinogen and FDPs were frequently observed in a dense and diffusely distributed fashion throughout the stroma (n = 9) and, in some cases (n = 2), in tumor cell areas. This extensive pattern of stromal deposition of fibrinogen/fibrin was also seen using PAbs to fibrinogen/fibrin.

Although tumor cell labeling for TF was found in only four of the squamous cell tumors, the TF in some of these tumor cells was clearly functional, as defined by the ability of the protein to bind biotinyl-FPR-ck-VIIa (Figure 4) and induce the deposition of cross-linked fibrin (Table 3). Similar labeling with the FPR-ck-VIIa probe was observed in TAMs in the squamous cell tumors (data not shown), suggesting that both the tumor cell and the macrophages were capable of activating blood clotting in these specimens.

## *Large-Cell Carcinoma*

Cross-linked fibrin was observed in an intimate association with endothelial cells in all five cases of large-cell carcinoma studied, as well as in association with tumor cells per se in two cases. TF was present in an endothelial distribution similar to that of fibrin in four cases and was positive on tumor cells in three, including both tumors that contained fibrin-positive tumor cells. CP was observed in one case on endothelial cells in conjunction with XLF and in one case on tumor cells in the absence of associated detectable fibrin deposits. Only minor deposits of FDPs were found; in two tumors this was associated with endothelial cells, and one tumor exhibited a diffuse deposition.

## *Small-Cell Carcinoma*

Both cases of small-cell carcinoma were associated with stromal deposits of XLF and TF-positive leukocytes,

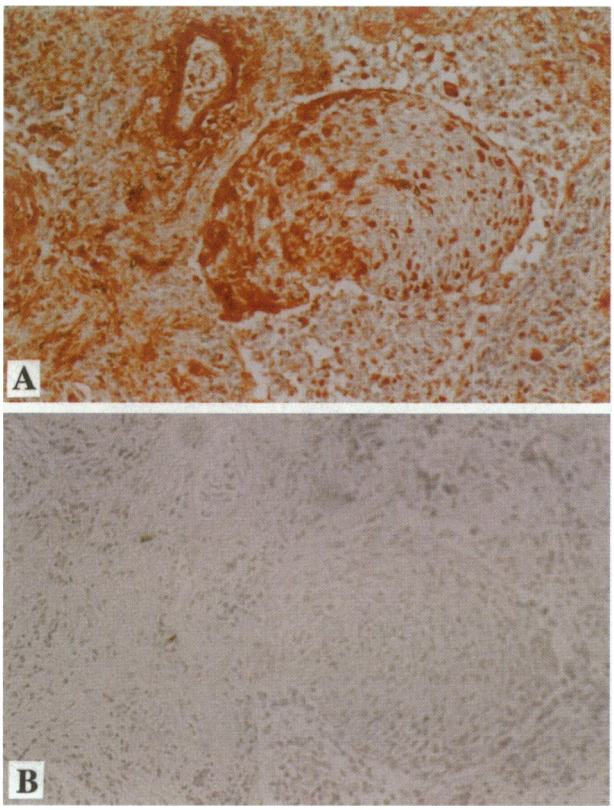


**Figure 4.** *In situ* localization of functional TF in squamous cell carcinoma of the lung. The binding sites on TF for factor VIIa were probed with biotinylated Phe-Pro-Arg-chloromethyl-ketone-labeled rVIIa (FPR-ck-VIIa), as described in Materials and Methods. As noted in **A**, the tumor nodule stains extensively for TF, indicating its capacity to bind factor VIIa and, therefore, its ability to activate clotting. The specificity of this reaction is demonstrated in **B** by the failure to stain with FPR-ck-VIIa after preincubating the tissue with a 50X molar excess of unlabeled rVIIa.

whereas tumor cells exhibited only limited and weak labeling for TF and no reactions for fibrin or CP.

In summary, deposits of XLF were found associated with the stroma of 20/25 (80%) lung tumors examined but was associated with the tumor cells in only 4/25 (16%). TF was found on macrophage-rich mononuclear cell infiltrates, endothelial cells, and fibroblasts within the stroma of 19 cases (76%), in addition to 7 cases of apparent tumor cell expression of TF (28%). Cancer procoagulant was detected much less often, being present in the stroma of 5 tumors (20%), particularly on endothelial cells, and on tumor cells in 3 cases (12%).

## *Breast Cancer*

As angiogenesis is typically more prominent in breast cancer than lung cancer, we turned our attention to the evaluation of human breast tumors. In a recently published study we demonstrated preferential expression of TF on tumor cells and vascular endothelial cells (VECs) in invasive human breast cancer (in association with XLF). No TF expression or XLF deposition was observed in benign breast disease.[22] These findings suggested that TF might be a marker for the switch to the angiogenic phenotype of the VECs and that, in human breast cancer, TF may be a pro-angiogenic protein.[41] Therefore, we examined an additional three cases of invasive breast cancer for co-localization of TF and VEGF and utilized *in situ* hybridization with oligonucleotides for TF to confirm the synthesis of TF by the tumor cells in one of the cases (Figure 5). As noted previously in our studies of lung cancer (Figures 2 and 3), TF and VEGF proteins co-localized prominently to breast tumor cells, as determined by immunohistological labeling (Figure 5, A and B), although TF staining was also observed in the TAMs and in the adjacent blood vessels in the VECs (data not shown). That the TF is synthesized by the tumor cell is supported by the finding of TF mRNA in the tumor cells by *in situ* hybridization (Figure 5C). Figure 5C shows TF-positive tumor cells lining ducts; figure 5D is a negative control. This interesting potential relationship between TF and VEGF, explored by others in murine tumors,[16] was examined more closely *in vitro* in human breast cancer cell lines. In preliminary studies of 10 human breast cancer cell lines (Table 2) we found a highly statistically significant correlation between the level of TF and VEGF ($r^2 = 0.84$; $P < 0.0001$; Figure 6). In control experiments (results not shown), we demonstrated that high-titer blocking antibodies to TF failed to suppress the synthesis of VEGF by the cells. Likewise, anti-VEGF blocking antibodies failed to suppress TF production by the cells. Neither antibody affected the growth of the cells *in vitro*. Taken together, these data support the hypothesis that TF up-regulates VEGF expression in human tumors by an intracellular mechanism and may, therefore, play a key role in tumor angiogenesis.

## *Discussion*

Although the data from an earlier study of the biochemical characterization of tumor procoagulants[21] suggested that the predominant PCA in human tumors is TF, the cell of origin of the PCA (ie, TAM *versus* tumor cell) remained uncertain. To obtain more precise detail of tissue localization, we employed a panel of well characterized MAbs for immunohistological localization of TF and CP *in situ* in human lung cancer in association with extravascular fibrin deposition.

Overall, relatively abundant deposits of XLF were observed in the stroma of 80% of the lung tumors examined but were detected in apparent association with tumor cells in only 16% of cases, ie, two cases of squamous cell carcinoma and two cases of large-cell carcinoma. In the latter, strands of XLF were seen to envelop and surround discrete tumor cells and nests of tumor cells. However, the overwhelming predominance of XLF deposition was in tumor stroma. Within the tumor stroma, fibrin was frequently localized in association with or in proximity to host mononuclear cells. Moreover, by morphological criteria and by binding of anti-monocyte/macrophage MAbs, the majority of labeled cells were mononuclear phagocytes. In addition to macrophages, and more distant to tumor cells, VECs and perivascular fibroblasts were also associated variably with XLF. The *in situ* localization of TF and XLF on the surface of TAMs is consistent with the results

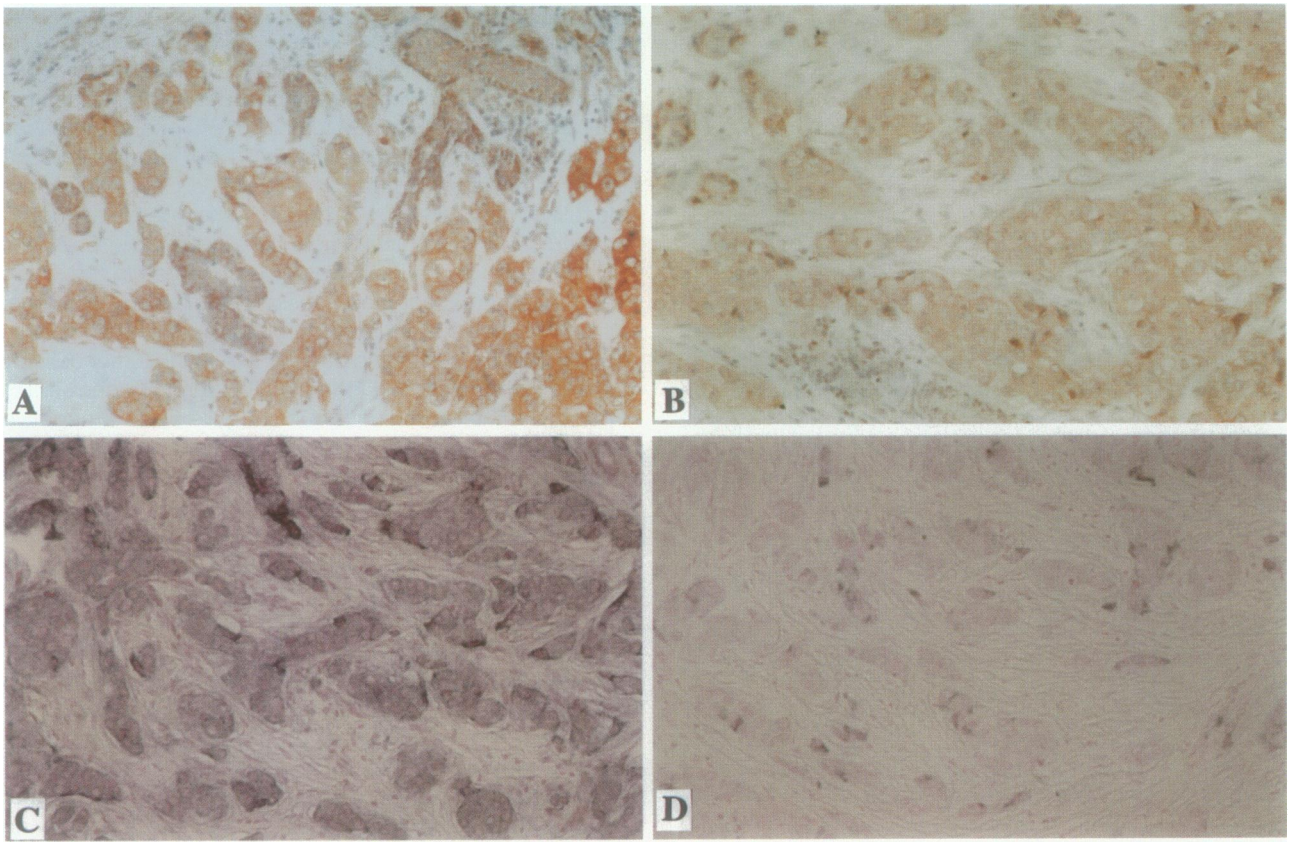


**Figure 5.** *In situ* localization of TF and VEGF in invasive human breast cancer. Invasive ductal breast carcinoma tissue was examined by immunohistochemistry for the presence of TF (**A**) and VEGF antigens (**B**), as described in Figure 2. TF synthesis by the tumor cells was confirmed by *in situ* hybridization (method 2), using a mixture of five overlapping biotinylated TF-specific riboprobes; the nonradioactive signal was detected using alkaline phosphatase covalently linked to streptavidin and the dye substrates NBT and BCIP, which form a purple signal. **C:** Positive tumor cells are seen lining the ducts. **D:** The negative control utilized a riboprobe for adenovirus 2; the positive control ($\beta$-actin) is not shown. Magnification, ×50.

of the functional studies of adherence-separated, tumor-associated cell subpopulations.[21]

Fibrin deposition surrounding tumors is presumed to be the result of a series of sequential events beginning with the development of leaky tumor-associated blood vessels. Studies of experimental tumor models in animals have shown that tumor cells produce one or more cytokines that increase capillary permeability for the extravasation of fibrinogen and other clotting proteins, promote monocyte and endothelial cell migration, and activate TF in both cell types.[8–12] Moreover, it is likely that VEGF and other permeability factors can act on adjacent normal tissues, exposing diffused clotting protein substrates to TF, with the latter expressed on the exposed surface of fibroblasts and histiocytes,[33] VEGF and/or TNF-stimulated VECs,[10,12] and activated monocytes and macrophages.[27,32,33,42–44] We and others have proposed that this sequence of events results in extensive deposition of fibrin at the interface between the tumor and normal host tissue.

Thus, TF was the predominant procoagulant molecule identified in this study and was found principally on peritumor inflammatory macrophages as well as scattered VECs and fibroblasts. TF expression by activated macrophages is a key feature of various inflammatory and delayed hypersensitivity reactions and has been implicated in the pathogenesis of extravascular fibrin deposition in a number of cellular immune processes.[5,45,46] Circulating monocytes in patients with cancer are known to express significantly increased levels of monocyte TF, consistent with an *in vivo* priming process.[47,48] Studies by Semeraro and colleagues have suggested that monocytes or macrophages recovered from body cavities adjacent to tumor growth are even more likely to demonstrate increased PCA, usually of the TF type.[49–51] Although the mechanisms responsible for induction of macrophage TF expression could not be determined in the current study, possibilities include a direct effect of tumor cells on macrophages[49] and/or stimulation of the host immune system with the subsequent release of TF-inducing cytokine(s) by activated T cells.[4,5,42–46,52,53]

We routinely found fibrin deposition in conjunction with TF expression in squamous cell and large-cell lung carcinomas. By contrast, adenocarcinomas were relatively devoid of extravascular fibrin formation, except in association with interstitial TAMs (Figure 2). All seven of the original cases lacked fibrin in relation to tumor cells, and only four cases exhibited stromal fibrin deposits; these deposits were limited to vascular or perivascular areas in two of the four. This is of interest because of the characteristically slower growth rate of adenocarcinomas, perhaps eliciting a less intense host response. Additional

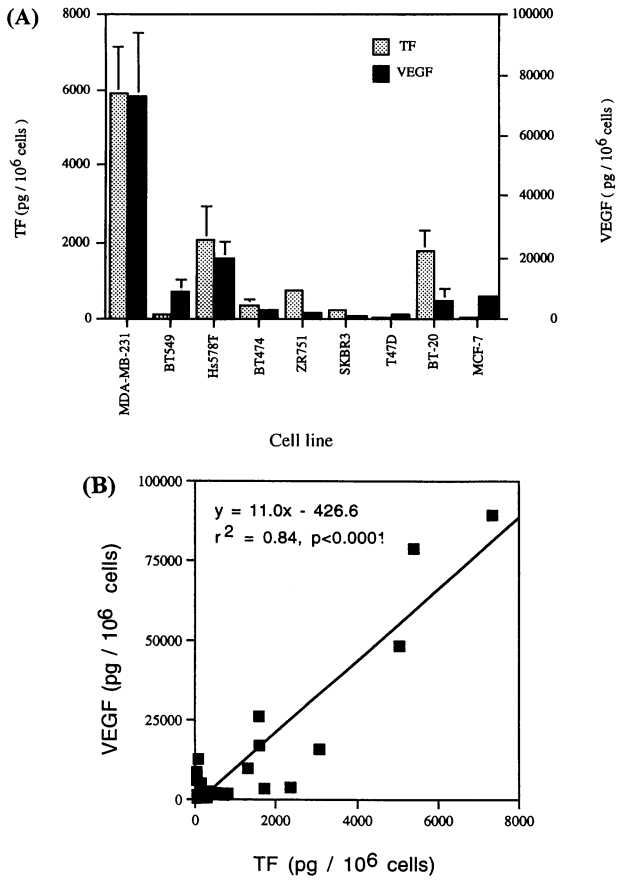


**Figure 6.** Relationship between the production of TF and VEGF in human breast cancer cell lines. **A:** Quantification of TF in sonicates of tumor cells and VEGF in tumor cell supernatants. **B:** Regression analysis for TF antigen *versus* VEGF antigen ($r^2 = 0.84$; $P < 0.0001$).

studies of larger numbers of this tumor type will be necessary to determine the validity of this observation.

In both cases of small-cell carcinoma available for the present study, TF and fibrin were confined to mononuclear cells in the peri-tumor stroma. These results differ from those of Wojtukiewcz et al,[54] who demonstrated a similar stromal distribution of XLF but emphasized the finding of TF on tumor cells in all six of their cases. Differences in results between these two studies cannot be immediately resolved but may indicate biological variability, or perhaps differences in the epitopes recognized by different antisera. This latter variable can be exacerbated by the use of different tissue fixation techniques, shown clearly in a previous comparison study between the two laboratories.[22]

In contrast to TF, CP antigen was only infrequently identified in this series of lung tumors. Cancer procoagulant was detected on tumor cells in only two cases of squamous cell carcinoma and in one case of large-cell carcinoma. No previous tissue localization studies of this factor-X-activating cysteine protease have been reported. However, by functional criteria, PCA attributable to CP has been demonstrated in cells from patients with malignant melanoma,[55] acute leukemia (both lymphocytic and nonlymphocytic),[56,57] and breast or colon cancer.[58]

Our findings of TF localized to the VECs of some of the tumors and co-localization of TF and VEGF in the tumor cells is of interest, particularly in the context of recent data linking TF expression in tumor cells and endothelial cells with angiogenesis.[16,22,41,59] Zhang and colleagues[16] demonstrated that hyperexpression of the TF gene in murine tumor cells that normally produce little or no TF results in cells that synthesize increased amounts of VEGF. When grown as syngeneic tumors, these cells stimulated enhanced angiogenic activity *in vivo*. In contrast, TF antisense sequences inhibited VEGF production in the cells and resulted in tumors with reduced angiogenic behavior *in vivo*.[16] In preliminary experiments we have extended these observations and documented a similar relationship between TF and VEGF in human melanoma cells grown as xenogeneic tumors in SICD mice.[59] In immunohistological studies of benign and malignant human breast disease,[22] we observed rather intense expression of TF in the VECs only in invasive malignant tumors of the breast. In view of the notorious resistance of human VECs to induction of TF *in vivo*,[60] we postulated that the neoangiogenic VECs in breast cancer might be phenotypically (or genotypically) different from normal VECs and may account for the increased thrombogenicity associated with breast cancer.[22,61] Furthermore, the strong quantitative relationship between the synthesis of TF and VEGF in human melanoma and breast cancer cell lines[59] (Figure 6) supports further the hypothesis that TF, in addition to its important role as a tumor procoagulant, may be a pro-angiogenic regulator in tumor cells as well as a marker for the switch to the angiogenic phenotype in tumor-associated VECs.[16,22,41,59] The mechanism for this cross-molecular regulation is as yet uncertain, although the role of TF in cell signaling events is under close scrutiny.[61]

In summary, the results of our study demonstrate that extravascular fibrin deposition is a frequent event in human carcinoma of the lung, generally in conjunction with local production of the procoagulant TF or, in occasional cases, CP. Moreover, over 75% of tumors showed co-localization of cross-linked fibrin and TF to stromal areas rich in host mononuclear cells, especially macrophages, consistent with a major contribution of activated macrophages to extravascular fibrin deposition in human carcinoma of the lung. Taken together with the functional characterization of TF as the predominant PCA expressed by suspensions of tumor cells and host cells,[21] these data are consistent with a major contribution of TF-rich, activated macrophages to extravascular fibrin deposition in human tumors. The importance of TF expression in the VECs of tumors is yet to be determined, but in breast cancer VEC TF appears to be a marker for the neoangiogenic response and is associated with fibrin deposition in the vessel wall.[22]

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